Clinical Chemistry 49:4
542–551 (2003)

Molecular Diagnostics and Genetics

Comparison of Two *CYP2D6* Genotyping Methods and Assessment of Genotype-Phenotype Relationships

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Background: **There have been no published reports comparing the CYP450 GeneChip® microarray assay with more standard methods of genetic testing.**

Methods: **We collected 20-mL blood samples from 236 volunteers for DNA isolation and testing before each individual ingested 60 mg of dextromethorphan, and collected their urine. *CYP2D6* alleles **3* to **7*, **9*, **17*, and **41*, and multiple *CYP2D6* gene copies were tested by allele-specific PCR (AS-PCR), whereas alleles **2* to **4* and **6* to **11* were tested by the Affymetrix CYP450 GeneChip assay. Five of the *CYP2D6* alleles (**3*, **4*, **6*, **7*, and **9*) were tested by both AS-PCR and the CYP450 GeneChip assay in an independent and blinded fashion in 232 of the 236 healthy volunteers. The combined *CYP2D6* genotype from both methods was used to divide the population into four subgroups, poor metabolizers (PMs), intermediate metabolizers (IMs), extensive metabolizers (EMs), and ultrarapid metabolizers (UMs), based on their relative function and ability to express the *CYP2D6* gene. The urinary elimination of dextromethorphan was assessed in each of these *CYP2D6* subgroups.**

Results: **The *CYP2D6*3*, **4*, **6*, **7*, and **9* alleles showed a high degree of concordance between the CYP450 GeneChip and AS-PCR methods (>99% concordance). The mean (SD) of the log[dextromethorphan metabolic ratio (MR)] in the four *CYP2D6* subgroups was PM = 0.49 (0.38); IM = −1.24 (0.53); EM = −2.35 (0.61); and UM = −2.43 (0.38).**

Conclusions: **Oligonucleotide microarray technology is an efficient and reliable way to test for *CYP2D6* gene variation based on five alleles compared by separate methods. The methodology is influenced by the quality and amount of DNA present. The log(dextromethorphan MR) is a highly variable index that appears to reflect the crude nature of the dextromethorphan MR as an indicator of CYP2D6 in vivo enzyme activity.**

A total of 67 point mutations and 9 insertions or deletions of ≥3 bp account for 43 cytochrome P450-2D6 (*CYP2D6*) alleles reported to date *(1)*. Many of the *CYP2D6* gene variations affect the expression or activity of the CYP2D6 enzyme to various extents. Interindividual variability in CYP2D6 enzyme activity observed within the general population ranges from complete absence of this enzyme to its overexpression. Gene expression in individuals with inactive *CYP2D6* alleles [poor metabolizers (PMs);[6] 5–8% of Caucasians] and individuals with multiple functional *CYP2D6* gene copies [ultrarapid metabolizers (UMs); 2–5% of Caucasians] are purported to define the two extremes in CYP2D6 enzyme activity within the population. However, among the vast majority of individuals

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Received October 4, 2002; accepted January 13, 2003.

[6] Nonstandard abbreviations: PM, poor metabolizer; UM, ultrarapid metabolizer; AS-PCR, allele-specific PCR; DXT, dextromethorphan; MR, metabolic ratio; SSPE, sodium chloride–sodium phosphate–EDTA; IM, intermediate metabolizer; EM, efficient metabolizer; and DRP, dextrorphan.

who express the CYP2D6 enzyme, its activity appears to show substantial variation as reflected by a typical metabolic ratio (phenotype) measurement. Some of this variation may be accounted for by a more complete *CYP2D6* genotype, but testing for numerous alleles in this gene remains a major undertaking today *(2)*. Thus, it is necessary to ask two questions: (*a*) can newer technology that determines genetic variation at multiple sites simultaneously reliably replace methods that assess genetic variation one polymorphism at a time; and (*b*) if an accurate and extensive assessment of large numbers of *CYP2D6* alleles can be done, what are its real merits or limits over a far less sophisticated and more direct assessment of CYP2D6 enzyme activity provided by a simple phenotype? To address these two issues, we compared the reliability of the microarray GeneChip® technology with allele-specific PCR (AS-PCR) methods at five major allelic sites on the *CYP2D6* gene. The relative potential of a genetic test for assessing in vivo CYP2D6 enzyme activity was also compared with the dextromethorphan (DXT) urinary metabolic ratio (MR) that has found broad use as an indicator of in vivo CYP2D6 enzyme activity.

Recent results from several laboratories have demonstrated that a limited *CYP2D6* allele set may predict the vast majority of Caucasian individuals lacking CYP2D6 enzyme activity with close to 100% accuracy *(2–8)*. As more *CYP2D6* null and reduced activity alleles have been discovered in various populations, the ability to account for more subtle variations in CYP2D6 enzyme activity has improved. Before focusing on how much *CYP2D6* genotype detail is required in clinical settings, a better understanding of the general reliability provided by new genetic testing methods and a relative appreciation of the limits inherent in the phenotype and genotype are needed.

Materials and Methods

PARTICIPANTS

As a part of the *CYP2D6* polymorphism inclusion criteria for a clinical study, a total of 236 healthy individuals were recruited for this research. The study protocol was reviewed by an independent ethics committee/Institutional Review Board for Bio-Kinetic Clinical Applications in Kansas City, Kansas. Volunteers were recruited from a single site, and each volunteer signed an informed consent before participating. A medical history was obtained from all volunteers to determine that there was no alcohol or drug abuse in the last year, no donation of blood or plasma in the last 30 days, no prescription or nonprescription drug use in the last 2 weeks, and no participation in any investigational drug study in the last 30 days. In addition, a urine drug screen was done at the time of recruitment to verify the absence of any drugs of abuse. Volunteers were asked to refrain from alcohol, coffee, tea, chocolate, and cola beverages for 24 h before the start of the study and through its completion. We first collected a 20-mL blood sample for *CYP2D6* genetic testing from each volunteer; volunteers then collected a blank urine sample before taking 60 mg of DXT and collecting all of their urine over the next 8 h in an unsupervised setting.

DNA ISOLATION METHODS

DNA was extracted from whole blood according to a previously described method at the University of Kentucky, College of Pharmacy *(4)*. Briefly, this method involves lysis of all blood cells with a hypotonic salt solution and Igepal CA630 detergent, followed by the centrifugation and collection of cell nuclei. The cell nuclei are lysed with a hypertonic salt solution, and the proteins are degraded in a proteinase K–sodium dodecyl sulfate solution. Residual proteins are precipitated by 6 mol/L NaCl, and the supernatant containing the DNA is transferred to a fresh tube. The DNA is precipitated with an equal volume of absolute ethanol and washed with 700 mL ethanol/L of water. The precipitated DNA is transferred to a clean, sterile Eppendorf tube to which 500 μL of Tris-EDTA buffer (pH 8.0) is added to reconstitute the precipitated DNA. In some instances, residual blood was extracted with the Qiagen Blood Kit for the GeneChip CYP450 assay (Qiagen Inc., Valencia, CA).

GENETIC TESTING

Consenting participants were tested for *CYP2D6* alleles by AS-PCR or PCR followed by restriction enzyme digestion at the University of Kentucky for the *CYP2D6*3*, **4*, **5*, **6*, **7*, **9*, **17*, **41*, **1xn*, **2xn/*35xn*, and **4xn* alleles based on published methods with some modifications *(4, 9–16)*. All of these genetic tests use *CYP2D6* intron-specific primers for the initial amplification, followed by either restriction digestion or a second allele-specific amplification for the allele test (Table 1). The genetic tests do not distinguish a **2xn* from a **35xn* allele, but both the **2xn* and **35xn* alleles are associated with overexpression of the CYP2D6 enzyme. The assignment of **41* may include several as yet undefined *CYP2D6*2*-related alleles. The **17* allele designation also includes the **40* allele. The **1* allele designation is a composite of **1* plus **13*, **15*, **16*, **18*, **22* to **27*, **33*, **34*, and **36* to **39*.

Multiple-copy *CYP2D6* alleles were first detected by amplifying across adjacent *CYP2D6* genes between exon 9 through exon 1 to generate a 9.5-kb amplicon. The subsequent tests assume that *CYP2D6* sequential genes are identical and that these are the only duplicated alleles of *CYP2D6*. The relative hybridization of amplicon fragments to the GeneChip supports the assumption that sequential *CYP2D6* genes are indeed duplicated copies of the same allele, not different *CYP2D6* alleles.

The execution of this work over a period of several years meant that it was not possible to always go back and retest every sample for every allele. For example, seven DNA samples containing the 1661C, 2850T, and 4180C point mutations and initially classified as **2* by the GeneChip were not available for later −1584G/C promoter analysis after this polymorphism was reported in

Table 1. Primers, primer pairs, amplicon size, allele test, and PCR conditions for all amplifications.

Primer name	Primer sequence	Primer pair	Amplicon size and/or allele test	PCR conditions
2D6F 2D6R	5′-GTGTGTCCAGAGGAGCCCAT-3′ 5′-TGCTCAGCCTCAACGTACCCC-3′	2D6R + 2D6F	4.4-kb amplicon	93 °C for 1 min; 93 °C for 1 min, 65 °C for 30 s, 68 °C for 5 min × 35 cycles; 72 °C for 10 min
UPF14 2D6PI1	5′-GCCTGGACAACTTGGAAGAACC-3′ 5′-GTGGTGGGGCATCCTCAGG-3′	UPF14 + 2D6PI1	1.9-kb amplicon	95 °C for 2 min; 95 °C for 15 s, 61 °C for 30 s, 72 °C for 2 min × 33 cycles; 72 °C for 10 min
AFFY-D12R UPF14	5′-GGTCCCACGGAAATCTGTCTCTGT-3′ 5′-GCCTGGACAACTTGGAAGAACC-3′	AFFY-D12R + UPF14	2.7-kb amplicon	95 °C for 2 min; 95 °C for 15 s, 61 °C for 30 s, 72 °C for 2 min × 3 cycles; 72 °C for 10 min
2D61BF 2D62AR	5′-GCATTTCCCAGCTGGAATCC-3′ 5-CCGGCCCTGACACTCCTTCT-3′	2D62AR + 2D61BF	1.8-kb amplicon	93 °C for 1 min; 60 °C for 30 s, 72 °C for 5 min × 28 cycles; 72 °C for 10 min
DFP DRP	5′-ACCGGGCACCTGTACTCCTCA-3′ 5′-GCATGAGCTAAGGCACCCAGAC-3′	DFP + DRP	3.5 kb (**5* allele test)	93 °C for 1 min; 65 °C for 30 s, 68 °C for 5 min × 35 cycles; 72 °C for 10 min
Lx2F Lx2R	5′-GCCACCATGGTGTCTTTGCTTTC-3′ 5′-ACCGGATTCCAGCTGGGAAATG-3′	Lx2F + Lx2R	9.5-kb amplicon test for *CYP2D6* multiple gene copies	94 °C for 1 min; 94 °C for 30 s, 68 °C for 12 min × 16 cycles; 94 °C for 30 s, 68 °C for 12 min + 15 s/cycle × 16 cycles; 72 °C for 10 min
P92-1R Lx2F	5′-CTCAGCCTCAACGTACCCCT-3′ 5′-GCCACCATGGTGTCTTTGCTTTC-3′	Lx2F + P92-1R with 9.5-kb amplicon in place of genomic DNA	264 bp (**1xn*, **2xn*, and **4xn* alleles)	94 °C for 2 min; 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1.5 min × 25 cycles; 72 °C for 10 min (**1xn* cut by restriction enzyme *Ban*II; **2xn* and **4xn* uncut)
P11-4F P124R	5′-TCAACACAGCAGGTTCA-3′ 5′-CTGTGGTTTCACCCACC-3′	P11-4F + P124R with 9.5-kb amplicon in place of genomic DNA	433 bp (distinguish **4xn* from **2xn* and **1xn* alleles)	94 °C for 2 min; 94 °C for 1 min, 53 °C for 1 min, 72 °C for 1.5 min × 20 cycles; 72 °C for 10 min (**4xn* cut by restriction enzyme *Hph*I; **2xn* and **1xn* uncut)
2D65FA 2D66FA	5-CTGCTAACTGAGCACA-3′ (wild type) 5′-CTGCTAACTGAGCACG-3′ (mutant)	2D65FA + 2D62AR and 2D66FA + 2D62AR with 1.8-kb amplicon	577 bp [test for **3* allele (2549Adel)]	94 °C for 1 min; 48 °C for 1 min, 72 °C for 1.5 min × 12 cycles; 72 °C for 10 min
2D67BR 2D68BR	5′-GGCGAAAGGGGCGTCC-3′ (wild type) 5′-GGCGAAAGGGGCGTCT-3′ (mutant)	2D67BR + 2D61BF and 2D68BR + 2D61BF with 1.8-kb amplicon	577 bp [test **4* allele (1846G→A)]	94 °C for 1 min; 48 °C for 1 min, 72 °C for 1.5 min × 12 cycles; 72 °C for 10 min
EF20 ER1 ER2	5′-TGGCAAGGTCCTACGCTTCC-3′ 5′-CTCACGCTGCACATCCGGAT-3′ (wild type) 5′-CTCACGCTGCACATCCGGAG-3′ (mutant)	EF20 + ER1 and EF20 + ER2 with 1.8-kb amplicon.	470-bp fragment [test **7* allele (2935A→C)]	94 °C for 1 min; 65 °C for 1 min, 72 °C for 1.5 min × 12 cycles; 72 °C for 10 min
2D6MTW 2D6MTT 2D6 T2R	5′-GCAAGAAGTCGCTGGAGCAGT-3′ (wild type) 5′-GCAAGAAGTCGCTGGAGCAGG-3′ (mutant) 5′-CAGAGACTCCTCGGTCTCTCGCT-3′	T2R + 2D6MTW and TR2 + 2D6MTT with 1.8-kb amplicon	470-bp fragment [test **6* allele (1707Tdel)]	94 °C for 1 min; 65 °C for 1 min, 72 °C for 1.5 min × 12 cycles; 72 °C for 10 min
2D69CF 2D610CF	5′-CTTCCTGGCAGAGATGGAGAA-3′ (wild type) 5′-CTTCCTGGCAGAGATGGAGGT-3′ (mutant)	2D69CF + 2D62AR and 2D610CF + 2D62AR with 1.8 kb amplicon	517-bp fragment [test **9* allele (2613–2615del)]	94 °C for 1 min; 54 °C for 1 min, 72 °C for 1.5 min × 12 cycles; 72 °C for 10 min
2D617WT 2D617MT 2D617F	5′-CCCGAAACCCAGGATCTGGG-3′ (wild type) 5′-CCCGAAACCCAGGATCTGGA-3′ (mutant) 5′-CCAAGGTTCAAATAGGACTA-3′	2D617F + 2D617WT and 2D617F + 2D617MT 4.4-kb amplicon	237-bp fragment [test **17* allele (1023 C→T)]	94 °C for 1 min; 60 °C for 1 min, 72 °C for 1.5 min × 15 cycles; 72 °C for 10 min
2D61496F 2D61496R3	5′-GCCTGGACAACTTGGAAGAACT-3′ 5′-GTGCCACCACGTCTAGCTTT-3′	2D61496F + 2D61496R3 with 1.9-, 9.5-, and 2.7-kb amplicons	203-bp fragment from 1.9-, 2.7-, and 9.5-kb amplicons [test for (−1584 C→G) for **2* and **4* alleles]	94 °C for 5 min; 94 °C for 30 s, 64 °C for 30 s, 72 °C for 30 s × 25 cycles; 72 °C for 10 min. Cut with restriction enzyme *Bsr*I (**2* cut; **41* uncut). Use of 9.5-kb fragment to generate 203-bp fragment to identify type of multiple copy (**2xn* or **41xn* allele)

the literature *(15, 16, 18)*. The remaining DNA samples that were available were used in an attempt to generate a 1.9-kb amplicon with the primers described in Table 1 *(16–18)*. All 1.9-kb amplicons generated were tested to assess the −1584G/C polymorphism in the promoter region of the *CYP2D6* gene. The 1.9-kb amplicon relies on the presence of a *CYP2D7P* gene conversion element within intron 1 of the *CYP2D6* gene. A few *2 alleles (~4%) do not contain this *CYP2D7P* gene conversion element in intron 1, and thus no 1.9-kb amplicon can be generated. This 4% of *2 alleles represents *2-related alleles containing the 1661C, 2850T, 4180C constellation of point mutations. All identified *2 alleles for which the 1.9-kb fragment was not generated and all samples that contained two *2 alleles were retested using a second reverse primer located in intron 2 of the *CYP2D6* gene (Table 1). This generates a 2.7-kb amplicon from all *CYP2D6* genes that was tested for the presence of the −1584G/C variation regardless of whether the *CYP2D7P* gene conversion element is present in the *CYP2D6* gene (Table 1). The regulatory regions of duplicated **2xn* samples were similarly evaluated. An amplicon of the *CYP2D6* regulatory region (203 bp) was generated directly from the 9.5-kb amplicon obtained from the duplicated *CYP2D6* allele. That amplicon was then tested for the −1584G variation to confirm the **2xn* allele assessment.

CYP2D6 alleles (**2*, **3*, **4*, **6*, **7*, **8*, **9*, **10*, and **11*) were tested by Roche Molecular Systems (Alameda, CA) and Affymetrix (Santa Clara, CA) with the GeneChip CYP450 assay, an oligonucleotide microarray hybridization method. Briefly, *CYP2D6* exons 1–9 and adjacent flanking regions were coamplified in a multiplex reaction together with amplicons encompassing exons 4 and 5 of *CYP2C19* with primers and amplification conditions provided by the manufacturer. Ten microliters of the PCR amplicon targets were treated with a fragmentation reagent for 20 min at 25 °C (DNaseI at ~0.1 U/μg of DNA) in the presence of 0.5 U of alkaline phosphatase (Roche Molecular Biochemicals) to remove excess deoxynucleotide triphosphates, followed by heat inactivation (95 °C for 10 min). The fragmented DNA amplicons were 3′-end labeled using 25 μmol/L fluoresceinated dideoxy-ATP (NEB) and 10 U of Terminal Transferase (Roche Molecular Biochemicals) at 37 °C for 35 min and heat inactivated for 5 min at 95 °C. The labeled fragmented target was hybridized to the GeneChip CYP450 probe array in 500 μL of 5× sodium chloride–sodium phosphate–EDTA solution [SSPE; 1× contains, per liter, 8.76 g of NaCl, 10 mL of 1 mol/L NaH_2PO_4, 2 mL of 0.5 mol/L EDTA (pH 7.4)] containing 1× Denhardt's solution, 0.5 mL/L Triton, 1 mmol/L hexadecyltrimethyl ammonium bromide (CTAB), and 1 nmol/L F1 fluoresceinated control oligonucleotide, and subsequently washed with 6× SSPE in the GeneChip Fluidics Station 400 according to automated fluidics protocols provided by the manufacturer. Fluorescence hybridization intensity images were collected using the GeneChip Scanner 50 and analyzed using proprietary analysis algorithms that compare perfect match and mismatch probe sets to determine a call at each polymorphic site.

Fifteen polymorphic sites within *CYP2D6* were assessed, and the combination of polymorphisms observed was reported as defined alleles according to standard nomenclature *(1)*. For example, the **10* allele was assigned when 100T, 1661C, 1039T, and 4180C were present, whereas the **4* allele was assigned when these polymorphisms were observed together with 1846A. Many *CYP2D6* allelic variants contain the **2A* constellation (G1661C, C2850T, and G4180C), and this allele was assumed in the absence of 1758T (**8*) or 883C (**11*). However, the *2 allele could be incorrectly assigned because the additional polymorphisms associated with the **2* allele designation include **12*, **14*, **19*, **20*, **21*, **28*, **29*, **30*, **31*, **32*, **35*, and **40*, which were not queried by any alternative methods. It is noteworthy that the nested AS-PCR test for the *17 allele was based on an AS-PCR test for the C1023→T point mutation and that all tests for the **17* allele were identified as *2 alleles by the GeneChip based on the constellation of 1661C, 2850T, and 4180C point mutations. The *CYP2D6*1* allele was assumed when all *CYP2D6* alleles tested by both laboratories were absent.

GENOTYPIC CLASSIFICATIONS

Participants were placed in one of four groups based on the number and activity of the combined *CYP2D6* alleles from both classic and GeneChip methods. Participants were classified as UM if they carried at least three functional copies of the *CYP2D6* gene (i.e., **1/*1xn*, **1/*2xn*, **2/*1xn*, or **2/*2xn*). Individuals were classified as PMs if they carried any two of the following *CYP2D6* alleles: **3*, **4*, **4xn*, **5*, **6*; **7*; **8*, or **11*. Individuals were classified as intermediate metabolizers (IMs) if they carried one *CYP2D6* allele associated with diminished CYP2D6 enzyme activity (**9*, **10*, **17*, or **41*) and one nonfunctional *CYP2D6* allele (**3*, **4*, **4xn*, **5*, **6*, **7*, **8*, or **11*) *(19)*. Genotypes that did not fall into one of these three categories (e.g., individuals identified as carrying one or two functional alleles, **1* and/or **2*) were by default placed in the efficient metabolizer (EM) group.

Five individuals who carried two partially functional alleles [**41/*41* (n = 3), **41/*17* (n = 1), and **41/*9* (n = 1)] were classified prospectively based on their mean log(MR) measurement [log(MR) = −1.16] relative to the UM [log(MR) = −2.43], EM [log(MR) = −2.35], IM [log(MR) = −1.24], and PM [log(MR) = 0.49] groups. Inadequate information was available in the literature to decide whether the CYP2D6 activity associated with two partially functional *CYP2D6* gene copies should relegate these individuals to the EM or IM group. To avoid introduction of bias into the statistical analysis, we excluded individuals with two partially active *CYP2D6* alleles from the initial analysis to determine how varia-

tions in the *CYP2D6* genotype were related to the CYP2D6 phenotype.

PHENOTYPES

Each volunteer was asked to collect a blank urine sample and then ingest 60 mg of DXT and collect urine over the next 8 h in an unsupervised setting. The urine volume measurements were available for all participants, and 45-mL aliquots were saved in polypropylene tubes and frozen (−20 °C) until analyzed for DXT and dextrorphan (DRP).

Urine was assayed for DXT and DRP by adding 60 μg of buprenorphine (internal standard) to 1000 μL of urine followed by 1.0 mL of acetate buffer (0.10 mol/L, pH 4.5) and 100 μL of freshly prepared β-glucuronidase solution (2000 U/sample). Samples were incubated at 37 °C for 12–18 h. After the incubation, the sample pH was adjusted to above pH 11 with 1 mL of sodium phosphate (pH 12) and 200 μL of 3 mol/L NaOH. The analytes were extracted into 10 mL of *n*-butanol–hexane (10:90 by volume), and then back-extracted into water by the addition of 300 μL of 0.01 mol/L HCl. A 50-μL aliquot of the aqueous phase was injected into the HPLC. The HPLC system consisted of an Alltech Platinum phenyl column [100 × 4.6 mm (i.d.); 3 μm] connected to a Kratos fluorescence detector (excitation at 228 nm with a 300 nm emission cutoff filter). The mobile phase consisted of acetonitrile–water (80:20 by volume) containing 10 mmol/L KH_2PO_4 (pH 4.0) and was pumped at a rate of 1.0 mL/min. Positive quality controls (previously tested urine samples) were included in each phenotyping run. Blank urine samples from each patient were used as negative quality controls to detect potential unknown interfering urinary peaks. The intra- and interday CVs were <10% for the DXT and DRP concentrations over the concentration range tested. The assay had lower limits of detection of 33 μg/L for DXT and 300 μg/L for DRP. The limits of quantification were 60 μg/L for DXT and 600 μg/L for DRP. The upper limits were 5.4 mg/L for DXT and 49 mg/L for DRP. In situations in which DXT concentrations fell below these limits, a larger sample (up to 5 mL) was used for analysis, pushing the limit of quantification to 15 ng/5 mL. Samples were diluted when urinary DRP concentrations exceeded the limits of the assay.

The DXT MR was used as an index of in vivo CYP2D6 enzyme activity. The urinary DXT MR was determined for each individual based on the following equation:

$$\text{MR} = \frac{\mu\text{moles of DXT in 0–8 h urine sample}}{\mu\text{moles of DRP in 0–8 h urine sample}}$$

DATA ANALYSIS

We compared log-transformed MR values between groups of different genotypes and DXT and DRP excretion amounts by one-way ANOVA (SAS program). Significance was defined as $P < 0.05$. Post hoc analysis was corrected for multiple comparisons by use of the Bonferroni method.

Results

ASSOCIATION OF THE *CYP2D6* GENOTYPE WITH THE DXT PHENOTYPE

Of the 236 participants, 206 (87.3%) were Caucasian, 13 (5.5%) were African American, 6 (2.5%) were multiracial, 4 (1.7%) were Hispanic, and 3 (1.2%) were Asian based on self-descriptions. The race of four other participants (1.7%) was undefined. We placed 229 of the volunteers in four genotype subgroups based on the number and purported activities of the *CYP2D6* alleles. The remaining seven volunteers had genotypes that were considered ambiguous with regard to genotype group: five samples had *CYP2D6* genotypes of **41/*41*, **41/*9*, or **41/*17*, and two samples could not be retested because the **2* allele in the promoter region prevented definitive subgroup assignment (**4/*2* and **2/*2*; e.g., EM or IM).

A comparison of the MRs for various *CYP2D6* genotypes is summarized in Fig. 1. A trend toward a smaller mean log(MR) (higher enzyme activity) is evident as the number of functional *CYP2D6* gene copies increases, but substantial scatter exists within the specific genotypes and overlap between genotype groups is apparent. When we placed individuals in only four major groups (UM, EM, IM, and PM) based on their genotype and anticipated relative expression of the CYP2D6 enzyme, the trend toward higher MRs with lower CYP2D6 expression became evident (Fig. 2A). Although there was still substantial variation within the groups, the overall difference in the group mean log[MR] values was significant (Fig. 2A; $P < 0.001$, one-way ANOVA). The mean log[MR] for each group was significantly different from that of all other groups except for the differences between the UM and EM groups, which failed to reach significance.

Among 13 individuals exhibiting a MR >0.3, 10 carried two nonfunctional *CYP2D6* alleles (combinations of the **3*, **4*, and **5* alleles). However, three individuals classified with only the *CYP2D6*1*, **2*, and **41* alleles were identified with MRs near the antimode separating the EM from the PM phenotype {0.339 [**2(41)/*2(41)*, DNA not available for retesting for −1584G/C polymorphism and thus not plotted], 0.365 [**41/*1*], and 0.529 [**41/*1*]}. The racial composition of these three individuals was African American, multiracial, and Caucasian, respectively. One Caucasian with a *CYP2D6* genotype of **4/*5* had a MR of 0.404, overlapping with the MR for the three individuals who appeared to carry only **1*, **2*, and/or **41* alleles. The Caucasian and multiracial DNA samples have since been retested for a broader array of *CYP2D6* alleles (virtually every allele between **1* and **41*), but the genotypes in both samples remained unchanged (**41/*1*; data not shown). We have no DNA remaining from the African-American sample to permit its retesting.

The 0–8 h urinary recovery of DXT differed 40-fold

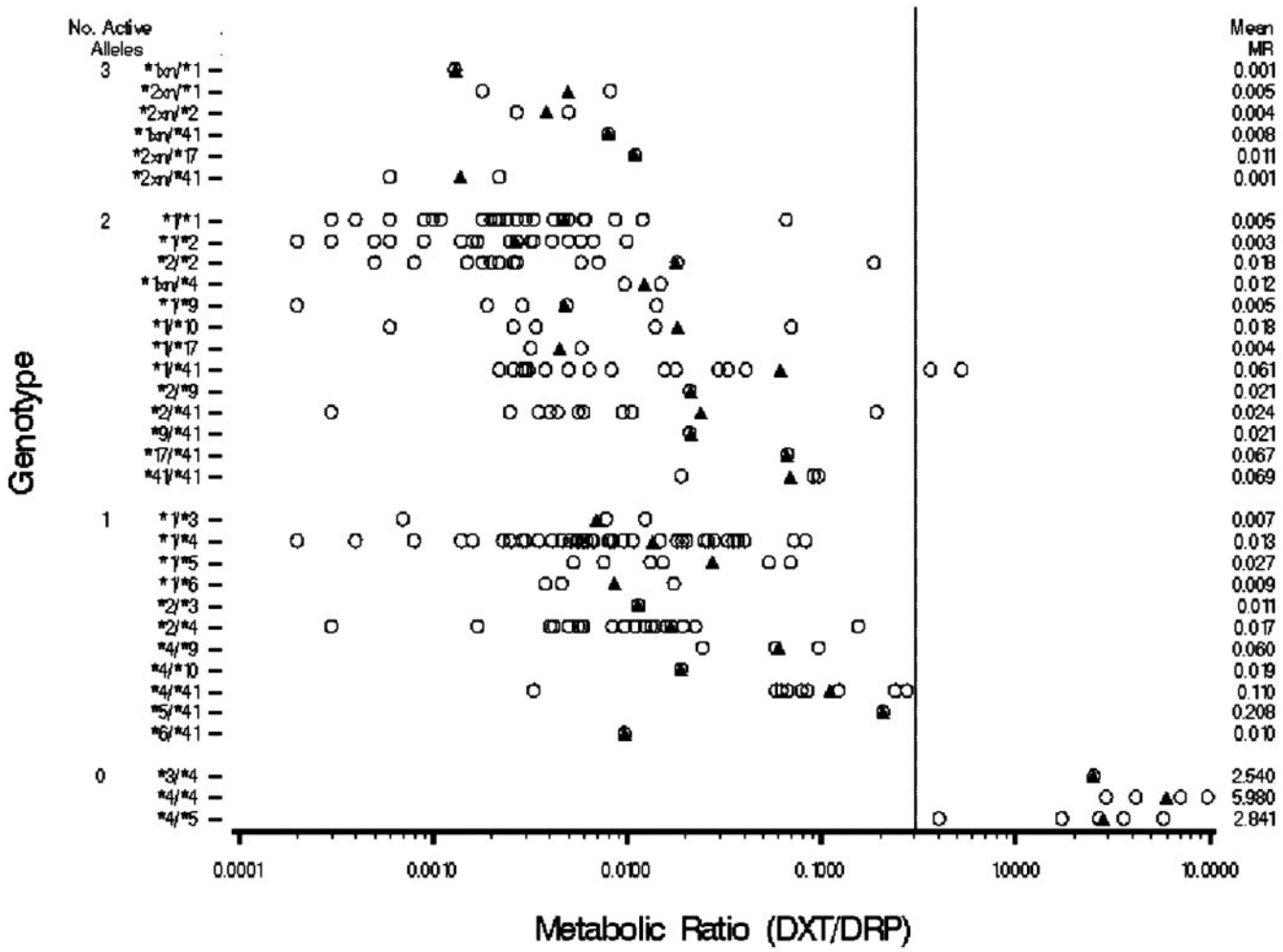

Fig. 1. Plot of *CYP2D6* genotype vs $\log_{10}$(DXT MR) for 229 individuals.

Seven samples that could not be retested for the −1584G/C promoter polymorphism were excluded from the plot. The mean MR for each *CYP2D6* genotype group is listed on the *right-hand y axis* and plotted as ▲. Genotype groups are divided into four separate categories based on 3, 2, 1, or 0 active (full or partially active) gene copies. The antimode separating the PM from the rest of the population is shown as a *dotted vertical line*.

between the *CYP2D6* genotype subgroups, with 3.2% of dose recoverable in the urine of the PM group and <0.008% in one individual with three or more functional *CYP2D6* gene copies (Fig. 2B; P <0.001, one-way ANOVA). The mean DRP excretion increased and then decreased as the number of functional *CYP2D6* gene copies exceeded two, with mean urinary recoveries of DRP for the PM, IM, EM, and UM groups of 0.93%, 18.6%, 26.6%, and 16.7%, respectively (Fig. 2C). The overall difference in DRP recovery among the groups was not significant when corrected for multiple comparisons.

CYP2D6 allele frequencies in us population

A summary of frequencies for the *CYP2D6* alleles tested in this study is provided in Table 2. The four inactive alleles observed in PMs (*CYP2D6*3*, **4*, **5*, and **6*) accounted for 24.2% (114 of 472) of the total alleles in this population. The remaining alleles associated with inactive enzyme expression (*CYP2D6*7*, **8*, **11*, and **4xn*) were not observed in this population. The frequency of alleles reported to be associated with diminished CYP2D6 enzyme activity (**9*, **10*, **17*, and **41*) was 15% (70 of 472) of the total alleles in the population. The frequency of the gene duplication alleles was 2.3% (11 of 472) of the total alleles in the population (Table 2).

comparison of *CYP2D6* genotyping by as-pcr with oligonucleotide microarray hybridization (Affymetrix CYP450 GeneChip)

We tested 232 individuals by AS-PCR and the Affymetrix CYP450 GeneChip assay in blinded fashion without previous knowledge of the genotype or phenotype of the individuals. In two individuals, the blind was broken before their analysis by the GeneChip. In one case, it was not possible to repeat the GeneChip analysis with the available DNA, and in the last case, the GeneChip could not call the genotype because the relative signal intensity

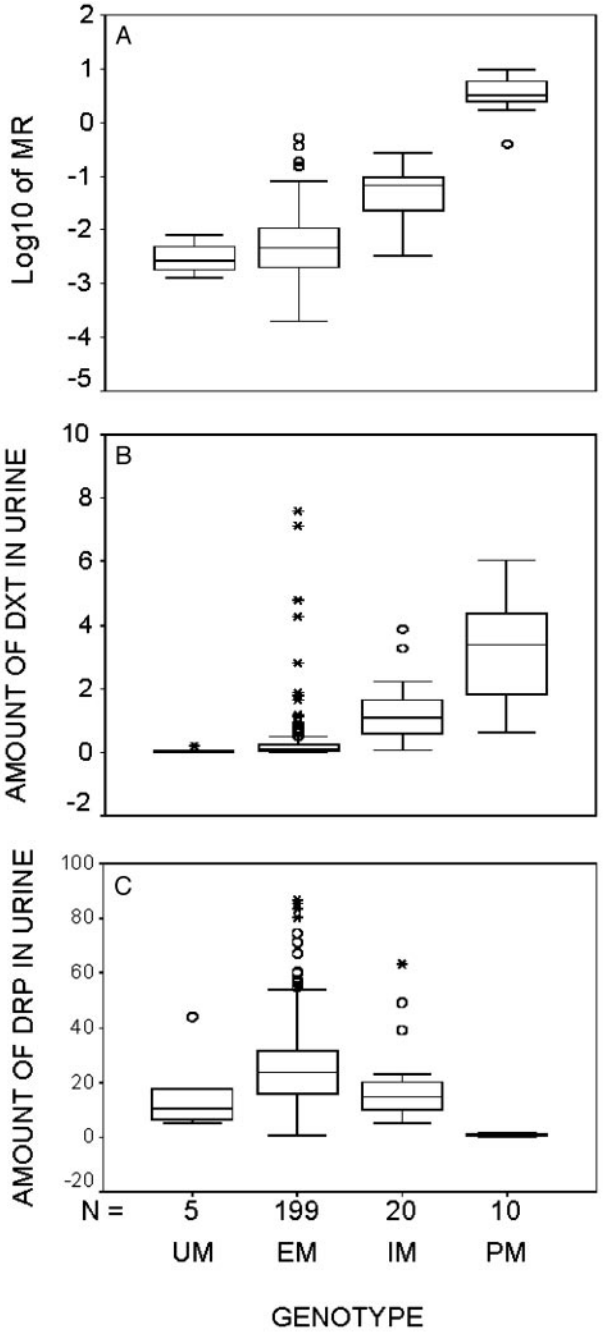

Fig. 2. Box plots of the $\log_{10}$(DXT MR) (*A*), urinary recovery of DXT (*B*), and urinary recovery of DRP (*C*) vs the *CYP2D6* genotype (*UM*, *EM*, *IM*, and *PM*) for 234 individuals.

Two individuals not tested for the −1584G/C polymorphism were excluded from the plots because the absence of that information influenced the group into which they would be placed (EM or IM). The *solid line* in each *box* represents the median value for each group. One-way ANOVA indicated that the differences between $\log_{10}$(DXT MR) and genotype group ($F = 84.3$; $df = 3,230$; $P < 0.0001$), between urinary recovery of DXT and genotype group ($F = 33.4$; $df = 3,230$; $P < 0.001$), and between urinary recovery of DRP and *CYP2D6* genotype group ($F = 10.2$; $df = 3,230$; $P < 0.001$) were significant after correcting for multiple comparisons. However, intergroup comparisons indicated that differences between the UM and EM groups were not significant in any of these plots and that in the DRP recovery plots, only DRP recovery in the PM group was significantly different from that of the other groups. Outliers (identified by ○ in each group) were not excluded from the analysis. ∗ represent very extreme outliers. *N* indicates the total number of individuals in each group and is the same for each plot.

Table 2. *CYP2D6* allele frequencies among the 236 US participants.

		Allele frequency	
Allele	Occurrence	All participants (n = 236)	Caucasians only (n = 206)
**1*	187	0.396	0.396
**2*	82	0.174	0.187
**3*	5	0.011	0.010
**4*	93	0.197	0.209
**5*	12	0.025	0.027
**6*	4	0.008	0.009
**7*	0	0	0.000
**8*	0	0	0.000
**9*	10	0.021	0.022
**10*	6	0.013	0.007
**11*	0	0.000	0.000
**17*	4	0.008	0.000
**41*	50	0.106	0.102
**1xn*	4	0.008	0.010
**2xn*	7	0.0148	0.010
**4xn*	0	0	0.000
Untested[a]	8	0.017	0.015

[a]Untested alleles were identified as containing point mutations at 1661C, 2850T, and 4180C, but were not available to retest for the −1584 point mutation in the *CYP2D6* promoter region.

of the second allele was too low with respect to the **1xn* allele.

The GeneChip CYP450 multiplex PCR reaction was sensitive to the quality of DNA used and initially failed to yield sufficient amounts of the longer PCR products in the multiplex reaction ~20% of the time (the seven multiplex products varied in size from 159 to 1125 bp—*CYP2D6* exons 1–2). In these cases, the use of smaller DNA volumes or DNA prepared with the Qiagen Blood Amp Kit (a minimum of 300 ng/reaction) was consistently found to support the multiplex reaction (data not shown). Table 3 summarizes the concordance between AS-PCR and the GeneChip assay. Genotyping results for the *CYP2D6*3*, **4*, **6*, **7*, and **9* alleles showed a high degree of concordance between the Affymetrix GeneChip and the AS-PCR methods. However, two discordant alleles were identified on comparison of the AS-PCR results with those of the GeneChip assay; both were associated with genotype errors by the AS-PCR method. During blind testing, the AS-PCR assay failed to detect a *CYP2D6*4* G1846→A splice site polymorphism in a heterozygous sample (**4/*1*). Reassessment of the gel picture indicated that the PCR amplification was weak, and retesting of the DNA sample two more times generated a consistent **4/*1* genotype. Another discrepancy resulted from an initial AS-PCR failure to identify a **9* allele in one sample (**9/*4*), an error that led to an initial misassignment to the EM instead of the IM group. This high concordance (>99%) has been typical with several subpopulations compared to date by both methods (W-H. Chou, F-X. Yan, T.B. Ryder, W-W. Liu, C. Perbost,

Table 3. Comparison of genotyping by AS-PCR with oligonucleotide microarray hybridization (Affymetrix GeneChip CYP450) in 232 individuals.[a]

	Total number of each *CYP2D6* allele called by PCR and GeneChip				
Methods	***3**	***4**	***6**	***7**	***9**
AS-PCR	4	93[b]	4	0	9[b]
GeneChip	4	94	4	0	10
Errors, n (%)	0/464[a] (0%)	1/464 (0.22%)	0/464 (0%)	0/464 (0%)	1/464 (0.22%)

[a]The GeneChip assay failed to call two samples; two more were not tested before breaking the study blind. One sample was not called because of limited amounts of DNA and the inability to reamplify after the initial seven-plex PCR failed. The second sample failed because of a multiple-copy *CYP2D6* allele coupled with a **4* allele. The results from these four samples were excluded from comparison. Genotypes of the four samples were as follows: **1xn/*4* (n = 1); **1/*4* (n = 1); **1/*3* (n = 1); and **1/*5* (n = 1) based on only AS-PCR methods. A total of 464 alleles were tested by both methods. The error calculation is based on the total false positives and false negatives in the sample set.

[b]Four **4* alleles were coupled with the **5* deletion and appear homozygous by the GeneChip assay and initial AS-PCR test. A **4* and a **9* allele were missed by the AS-PCR method.

M. Fairchild, J. de Leon, W.H. Koch, and P.J. Wedlund, unpublished results).

Discussion

comparison of *CYP2D6* genotyping by as-pcr with oligonucleotide microarray hybridization (Affymetrix GeneChip CYP450)

Genotypes of 232 individuals obtained by AS-PCR and Affymetrix GeneChip CYP450 were compared with >99% concordance between these methods for the five allelic variants tested (Table 3). Four individuals were excluded from the comparison because of unsuccessful DNA amplification (**1/*3*; DNA quality/quantity for the GeneChip assay) or an inability to obtain an unambiguous genotype by the GeneChip assay (**1xn/*4*). The remaining two genotypes (**1/*5* and **1/*4*) were eventually run and correctly identified, but not before the unblinding of the DNA samples; thus, they were omitted from the comparison. Only *CYP2D6*3*, **4*, **6*, **7*, and **9* alleles were included in the comparison because only these five alleles were detected by both assays at the time this study was initially conceived. Because it is important to consider false negatives and false positives in the error estimate, the total error was calculated based on the entire number of samples tested by both methods, not simply the sample number that contained just these alleles.

The Affymetrix GeneChip CYP450 assay and AS-PCR methods performed similarly in the current study. Historically, the error rate with oligonucleotide arrays has been similar to that of AS-PCR. We have identified discordant results between these two methods with other sample sets, which underscored the need to validate the oligonucleotide microarray system as thoroughly as any other new assay before assuming that it will perform flawlessly under all conditions. Some of the weaknesses of the oligonucleotide array are relatively easy to address; in other cases, the weaknesses could require changes in the oligonucleotides that are used in the microarray. For example, in an early modified research version of the assay, errors were made in calling some **4* alleles and a **3* allele that were traced to the PCR procedures. These problems were corrected in the final product version of the PCR multiplexing amplification. However, the seven-plex PCR reaction remains sensitive to the quality and quantity of DNA used, with the longer PCR products generated being the ones most adversely affected by these variables. A **4* allele (e.g., **4E*) call was complicated by difficulty in assessing the T3975C polymorphism. That problem was eventually eliminated by ignoring the **4E* subtyping polymorphism in the *CYP2D6* gene because all **4* variants are null alleles. The annealing of primers to certain oligonucleotide probes on the microarray does not always follow the expected or desired pattern for the homozygous and heterozygous sites. There is currently no method to reliably predict oligonucleotide hybridization behavior or avoid every type of problem that might arise with large numbers of oligonucleotide probes until they are evaluated with real DNA samples. On the positive side, once oligonucleotide probes have been tested and validated, their incorporation into new or different microarrays is straightforward and does not require further optimization if assay conditions are kept constant.

The GeneChip hybridization intensity patterns were also affected in samples with multiple copies of the *CYP2D6* gene, an issue that was identified only after several samples with multiple gene copies were tested by both methods. The GeneChip software failed to make a genotype call of some multiple-copy alleles, necessitating one or more retests of the sample. One DNA sample could not be called even after three separate tries (**1xn/*4*). The problem was traced to the base-calling algorithm cutoffs that were trained with single *CYP2D6* gene copy allele samples. Because a skewed hybridization intensity signal is generated by the nonduplicated allele at certain mutation sites in the *CYP2D6* gene relative to those arising from the multiple-copy allele, the signals sometimes fell outside of the cutoff range established for typical single gene copy biallelic distributions. This was not a frequent problem, but it did sometimes cause the assay to fail data quality tests performed by the analysis software. As such it was recognized as a technical issue that could be

resolved either by retraining the analysis algorithm or by assay design changes. A newer version of the CYP450 microarray assay that directly identifies the presence of gene duplications has been developed at Roche Molecular Systems and appears to have eliminated this problem. The newer CYP450 array and assay was not used in this research so that consistency could be maintained throughout the evaluation process.

Considering the magnitude of the variation that exists in the *CYP2D6* gene, the high-throughput, information-rich oligonucleotide microarray is a very practical solution to the more traditional genotyping approaches that typically assess one polymorphism at a time (e.g., restriction fragment length polymorphism analysis, AS-PCR, TaqMan®) *(2, 4)*. To acquire the same information obtainable from a single GeneChip assay requires far more work and labor and remains too slow and/or expensive to justify in a routine clinical environment. It currently takes ~4 days to do the separate long amplifications, nested PCR, and retests of amplicons to generate a limited *CYP2D6* genotype by the AS-PCR methods used by our laboratory. As the number of *CYP2D6* alleles tested has increased, so has the time required for their detection. In contrast, the microarray can detect all the alleles it tests simultaneously in a single day. Looking to the future, where it may be necessary to assess multiple genetic variations in multiple genes, informative multilocus screening technology (like that of the oligonucleotide microarray) may represent the only practical way to rapidly examine the genetic variation that exists in a population at important gene loci.

There are two caveats to this notion. One is that the technology for GeneChip analysis remains fairly expensive, and the other is that the CYP450 GeneChip itself is not inexpensive. There thus must be a fairly strong incentive for clinical laboratories to make this investment. Its advantage is the level of genetic detail it can provide within a relatively modest length of time and with a significantly reduced investment of human effort. Moving forward, researchers should be aware that oligonucleotide microarrays are not foolproof and that it will require more than just a cursory effort to establish their reliability and validate performance. Some of the limitations of microarray technology are not apparent until after many different genomic sample types have been tested from random groups of individuals.

CYP2D6 allele frequencies in the US population

The *CYP2D6* allele frequencies in this US population were similar to those found in previous European and US studies *(2, 7, 8, 20, 21)*. Four inactive *CYP2D6* alleles (*CYP2D6*3*, **4*, **5*, and **6*) accounted for a total of 24.2% of the total *CYP2D6* alleles vs 25.98% in European populations and 24.5% in other US groups. We did not detect other alleles, including *CYP2D6*7*, **8*, **11*, and **4xn* (associated with inactive enzyme expression), in this study group. Genetic testing for the four inactive alleles of the *CYP2D6* gene (**3*, **4*, **5*, **6*) accounted for nearly all individuals classified by the DXT MR as exhibiting the PM phenotype. Testing for this reduced *CYP2D6* allele set is probably adequate for most research done in Caucasians, but eventually a higher confidence level will be required if *CYP2D6* genetic testing finds its way into the therapeutic arena or is used in other racial groups.

Purists may point out that use of a MR to separate an EM from a PM for all races is open to question. However, it should also be noted there has been considerable intermingling of races in the US and that attempts to relate phenotypes and genotypes separately for Caucasians, African Americans, Hispanics, and Asians is both artificial and inappropriate. If the *CYP2D6* genotype can accurately predict CYP2D6 enzyme activity, it should be expected to be predictive of that enzyme activity regardless of the ethnic group. Although this implies that more alleles must be characterized within multiracial populations, that characterization will probably be necessary anyway if genetic testing is to find use as a therapeutic tool in today's multiracial society. Although the frequency of the *CYP2D6* alleles in just Caucasians is reported in Table 2, this was done because the mixture of racial groups in this subpopulation was not representative of the entire US population.

CYP2D6 genotype vs CYP2D6 phenotype

The DXT MR is a rough indicator of in vivo CYP2D6 enzyme activity, as suggested by the marked variation in this phenotypic characteristic in nearly every *CYP2D6* genotype (Fig. 1). The DXT MR is also adversely affected by virtually the same urinary recovery of the major metabolite DRP in the IM and UM groups. The need to administer a probe drug and to collect urine over the ensuing 8 h before defining CYP2D6 enzyme activity has always limited the practical utility of a patient phenotype. This time-consuming, patient- and therapy-dependent procedure has never found very widespread clinical acceptance, and it provides only descriptive information about a patient under a specific set of clinical conditions. Fortunately, the *CYP2D6* genotype is a fairly reliable predictor of CYP2D6 control enzyme expression and activity, at least for most individuals with the greatest extremes in CYP2D6 enzyme activity [Figs. 1 and 2; Ref. *(18)*]. The fact that a *CYP2D6* genotype can be assessed directly from blood or other biological samples makes the *CYP2D6* genotype a far more attractive way to characterize potential CYP2D6 enzyme expression in clinical settings. One advantage of the *CYP2D6* genotype lies with the realization that it is unaffected by the clinical environment. Whereas CYP2D6 enzyme activity may vary for one clinical reason or another, the *CYP2D6* genotype provides a point of reference from which to understand differences in CYP2D6 enzyme expression among patient that are not possible with the more descriptive phenotype assessment.

There has been substantial interest in characterizing CYP2D6 in vivo activity through a more detailed *CYP2D6*

genotype *(2, 5, 6, 8, 15, 18, 19)*. This work sheds additional light on those efforts and supports the use of a *CYP2D6* genotype to characterize individual CYP2D6 phenotypes as PM, IM, EM, or UM. However, it is the clinical environment where the actual potential of a *CYP2D6* genotype must be demonstrated *(22)* and eventually assessed for its ability to predict therapeutic outcomes and influence patient care costs. It is the impact of the *CYP2D6* genotype on outcome and costs that will ultimately define its true therapeutic utility *(23)*. Before focusing too much effort on finer and finer subdivisions of the *CYP2D6* genotype, it might be better to focus on whether the more extreme variations in this enzyme can be shown to actually affect clinical outcomes in patients, not just its ability to affect the dose, drug concentrations, or a specific side effect. These more limited efforts have dominated much of the clinical focus on this and other genetic polymorphisms to the detriment of efforts to determine whether genetic testing could actually serve as a viable therapeutic tool.

We would like to thank Bonnie Fijal for help with the plotting routine for the genotype/MR plots and Run-Mei Pan for contributions to the genetic testing work. This research was supported in part by Hoechst Marion Roussel, Inc. (now Aventis Pharmaceuticals, Inc.), which directed and performed all the clinical research and provided nearly all resources for the cost of the phenotype and genotype tests performed at the University of Kentucky laboratories. Roche Molecular Systems, Inc. and Affymetrix provided GeneChip testing for *CYP2D6* at no cost to the study and some PCR supplies. Dr. P.J. Wedlund is a paid consultant of Roche Molecular Systems, Inc.

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